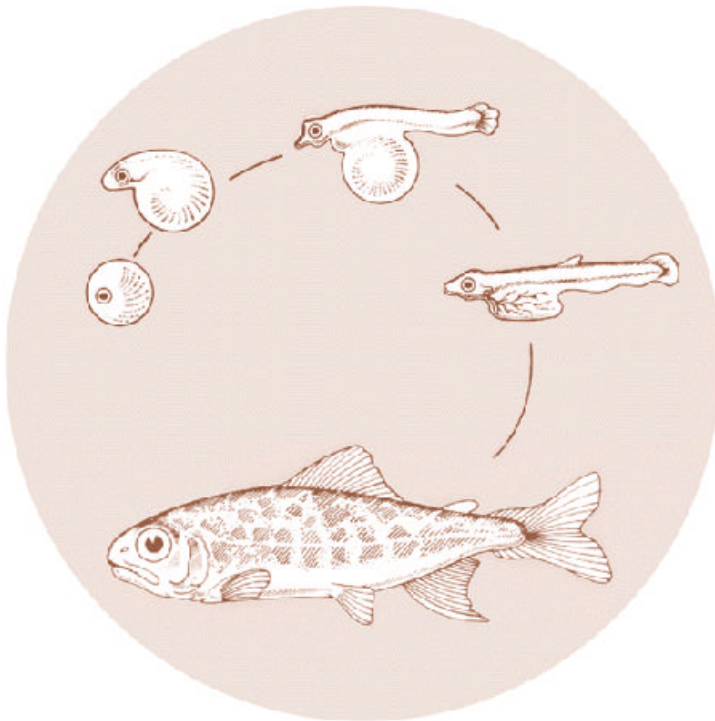


June 1986

EFFECTS OF VITAMIN NUTRITION ON THE IMMUNE RESPONSE OF HATCHERY-REARED SALMONIDS

Annual Report FY 1986



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EFFECTS OF VITAMIN NUTRITION ON THE IMMUNE RESPONSE
OF
HATCHERY-REARED SALMONIDS
ANNUAL REPORT FY 1986

by
Dave Leith, Project Leader
John Holmes, Project Biologist
Abernathy Salmon Culture Technology Center
Longview WA 98632

and

Dr. Stephen Kaattari, Project Leader
Ms. Mary Yui, Research Assistant
Department of Microbiology
Oregon State University
Corvallis, OR 97331

Prepared for
Dr. Gerald R. Bouck; Project Officer
Bonneville Power Administration
Division of Fish and Wildlife
P.O. Box 3621; Portland, OR 97208

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EXECUTIVE SUMMARY

The Abernathy Salmon Culture Technology Center (A.S.C.T.C.) and the Department of Microbiology at Oregon State University, with funding from the Bonneville Power Administration, are presently conducting a study on the effects of vitamin nutrition on immunity and disease resistance in chinook salmon (Oncorhynchus tshawytscha). It is the long range goal of this project to define the dietary levels of pyridoxine, folic acid, pantothenic acid, riboflavin, ascorbic acid and vitamin E required to assure maximum resistance to disease.

In the second year of this study, we have completed two preliminary range-finding studies on the vitamins, pyridoxine and folic acid. These studies allowed us to focus in on the concentration of these vitamins which would exert the greatest effects in a practical ration. Upon the resolution of these concentrations, we chose an appropriate vitamin concentration range for both vitamins within a practical ration (Abernathy diet) and a semi-purified ration (modified Oregon Test Diet). This would then enable us to determine what concentration of vitamin would be required to gain some beneficial effects in a practical feed, and to determine the absolute requirements for immunological sufficiency.

Results demonstrate that no significant differences in the specific growth rate or gross feed efficiency occurs, regardless of the concentration of either vitamin in the diets. This observation was made for both the preliminary range-finding trials as well as the formal diet trials.

The immunological assays, however, demonstrated some consistent differences with the pyridoxine diets. In both the preliminary and, thus

far, in the formal diet trials, mid-range concentrations (60-75 mg / kg diet) appear to give the greatest degree of immunostimulation.

Consistently observed modulation of the immune response by variation of the folic acid concentration has not been observed.

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INTRODUCTION

The presence of infectious diseases are one of the most important problems affecting the efficiency and economics of salmonid culture. Total mortality experienced during the egg, fry, and fingerling stages of Pacific salmon (Onchorhynchus spp.) culture, for example, normally ranges from 15-20% (Hublo and Jones, 1970). About 50% of that mortality occurs during the fry and fingerling phases, and most of those deaths are probably attributable to infectious disease. This results in the loss of many millions of fish each year. Additionally, mortality can significantly exceed normal ranges in the event of epizootics resulting in even larger losses during artificial propagation.

Diseases can also produce debilitated fish or survivors which harbor latent infections. Low-grade or latent infections have the potential for significantly reducing the post-release survival of smolts from hatcheries. For example, Sanders (1979) and Banner et al. (1983) have found that coho salmon (O. kisutch) fingerlings, suffering a low-grade infection of bacterial kidney disease (R. salmoninarum), died at high rates after transfer to seawater. Also, Wedemeyer et al. (1976) reported similar effects when coho smolts had subclinical cases of furunculosis (Aeromonas salmonicida).

Considerable research has been done to better understand fish diseases and develop means for their prevention and treatment. That work has concentrated primarily upon identification of agents causing disorders, improving diagnostic methods, exploring chemotherapeutic treatments, and developing vaccines. Very little work, however, has been done to determine the relationships among the nutritional

state, immunocompetence, and disease resistance in fish (Bell et al., 1984; Blazer and Wolke, 1984; Hardy, 1979; Durve and Lovell, 1982).

Research with livestock, laboratory animals, and humans has provided a large body of information which demonstrates the importance of host nutrition in combating disease. Observations made by pathologists at hatcheries indicate this is also true for fish. For example, Wood (1974) reports case histories in which juvenile coho salmon fed either a dry diet or a moist pellet containing corn gluten meal suffered higher mortality from bacterial kidney disease than did fish receiving the standard moist pellet formula. Wedemeyer and Ross (1973) could not confirm that corn gluten meal increased kidney disease mortality, but fish fed the corn product exhibited a more severe, nonspecific, stress-response to the infection.

Many vitamins have significant roles in the functioning of immune systems in man and other animals. Several researchers and reviewers (Axelrod and Traketelelis, 1964; Beisel, 1982; Blalock et al., 1984; Cunningham-Rundles, 1982; Debes and Kirksey, 1979; Scott et al., 1976; Wilgus, 1977) have reported that deficiency of pyridoxine suppresses the immune response in a number of animals. The requirement is dependent, in part, on the amount of dietary protein (Scott et al., 1976). Hardy et al. (1979) found that increased pyridoxine in a high-protein diet rendered chinook salmon fingerlings more resistant to Vibrio anguillarum, but additional pyridoxine in a low-protein feed did not have the same effect. Cell mediated responses have been demonstrated to be dramatically affected by reduced pyridoxine, as evidenced by depressed mixed lymphocyte reactivity, prolonged allograft survival, and reduced skin hypersensitivity (Axelrod and

Traketellis, 1964). Numbers of blood lymphocytes are dramatically reduced, as well as the weight and size of the spleen and thymus during pyridoxine deficiency (Debes and Kirksey, 1979). Antibody formation is decreased as determined by bacterial agglutination and plaque forming cell responses in rats, swine, chickens and humans fed diets deficient in pantothenic acid (Beisel, 1982; Nelson, 1978; Panda and Combs, 1963; Scott et al., 1976), however, that vitamin apparently has little effect on cell mediated immunity. Insufficient amounts of dietary riboflavin have also caused decreased antibody responses to S. pullorum in chicks (Beisel, 1982). Deficiencies of folic acid lead to reduced host resistance to salmonella and impaired humoral and cellular immune function in both man and experimental animals (Beisel, 1982; Cunningham-Rundles, 1982; Scott et al., 1976; Siddons, 1978). Impaired functioning of phagocytes and reduced response to phytohemagglutinin have also been observed in vitamin B12 deficiency states. Inadequate biotin intake in rats causes a reduced hemagglutinating antibody response to diphtheria toxoid, and reduced development of splenic plaque-forming cells after inoculation with sheep red blood cells (Beisel, 1982;). The amount of ascorbic acid in the feed has reportedly influenced disease susceptibility in several animals (Chatterjee, 1978). Durve and Lovell (1982) found that channel catfish (Ictalurus punctatus) were more resistant to the bacterium Edwardsiella tarda when fed elevated doses of vitamin C and the effect was more pronounced at lower water temperatures. On the other hand, Bell et al. (1984) had equivocal results when studying the effects of dietary ascorbate on the development of bacterial kidney disease in sockeye salmon (O. nerka). The role(s) performed by this vitamin are still unclear and controversial, however indications are that it may play an important

part in the normal functioning of phagocytic cells (Beisel, 1982; Cunningham-Rundles, 1982). Two fat soluble vitamins, A and E, have recognized effects on immune systems of other animals. Vitamin A probably influences resistance to infections through its role in maintaining the integrity of the epithelial and mucosal membranes as well as affecting humoral and cell mediated immune responses (Beisel, 1982; Pandal and Combs, 1963). Vitamin E has been shown to improve the humoral immune responses of mice, chicks, turkeys, swine, sheep, and guinea pigs when challenged with either nonliving antigens, living bacteria, or live viruses (Beisel, 1982; Colnago et al., 1984; Ellis and Vorhies, 1976; Heinseling et al., 1974; Nockels, 1980). Blazer and Wolke (1984) found that rainbow trout (Salmo gairdneri) showed a significantly reduced humoral immune response and reduction of several non-specific resistance factors when fed diets deficient in Vitamin E. Since Vitamin E is an antioxidant which prevents autooxidation of lipids and/or protects the animal from toxic effects of oxidation products (Lee and Sinnhuber, 1972), the amount of lipid in the diet and its degree of rancidity could be important factors in the relationship between this vitamin and immunity.

Past research to define the quantitative vitamin needs of juvenile salmonids has concentrated primarily on determining requirements for maximum growth and most efficient feed utilization (Halver, 1972; National Academy of Sciences, 1973) without attempts to assess the immunocompetency of test animals. Work is needed to determine the quantities of key vitamins required to insure optimal functioning of immune systems and high resistance to common diseases.

EXPERIMENTAL DESIGN

The vitamins chosen for study were pyridoxine, pantothenic acid, riboflavin, folic acid, Vitamin E, and ascorbic acid. A practical fish feed formulation and a semi-purified test diet are to be compounded to contain incremental amounts of the test vitamins. Each vitamin will be studied singly while dietary levels of all other vitamins are held constant, using the supplementation rates specified in the vitamin packages for each diet. The test diets are fed for up to 24 weeks to duplicate lots of spring chinook juveniles held indoors in circular tanks supplied with well water. During rearing, each lot of fish is weighed biweekly and dead fish are removed and recorded daily to: (1) determine growth rates, (2) calculate food conversion efficiencies, (3) provide population weights on which to base feeding rates, and (4) determine survival rates. Samples of test feeds are analyzed regularly to verify dietary concentrations, and fish organs will be analysed for vitamin levels at the termination of each study. Laboratory assays are designed to assess the degree of immune competence in fish from each diet group at regular intervals. The assays will be used to examine in vivo and in vitro parameters of humoral immunity, cellular immunity, phagocytic activity, and disease resistance. Data concerning growth, food conversion efficiencies, rearing mortality, times to death in disease challenges, and in vitro assays of immune responsiveness are tested by analyses of variance, regression techniques, and ranking to determine significance of differences (5% level of significance) and to calculate minimum amount of each vitamin required in the diet to insure maximum functioning of the immune system.

MATERIALS AND METHODS

Facilities. The feeding and rearing phases of the experiments were performed at the Abernathy Salmon Culture Technology Center (SCTC), Longview, WA. Immunological assays and disease challenges were performed on chinook salmon obtained from Abernathy and housed at the Oregon State University Department of Microbiology and the Fish Disease Laboratory (OSUFDL), Corvallis, OR.

During rearing, the fish were held in 1200-liter steel, circular tanks furnished with constant temperature (12°C) well water. During disease challenges, fish were housed in 30 liter fiberglass tanks supplied with 12°C well water. The compositions of the two water sources are given in Table 1.

The amount of water flowing into each 1200-liter rearing tank was maintained at 12 liters per minute throughout the studies. The 30 liter tanks used to hold fish during disease challenges received flows of 0.40 liters per minute. Fish population densities ($\text{kg}/\text{inflow}/\text{min}$ and kg/m^3 of space) never exceeded the guidelines of Banks et al. (1979)

Illumination of the experimental tanks was provided from fluorescent lights controlled by a photocell system to simulate the natural photoperiod at the latitude of the Abernathy SCTC.

Animals. Spring chinook salmon were used as test animals in all experiments. They were obtained as eyed eggs which had been collected from adult fish spawned at Carson National Fish Hatchery located about 15 miles north of Carson, WA on the Wind River. Eggs were transferred to the Abernathy Center, cushioned in moist burlap bags, supported in 40 cm x 40 cm x 25 cm egg baskets in order to prevent physical trauma. Upon arrival at the Abernathy S.C.T.C., the eggs were surface disinfected

Table 1. Composition of water supplies.

Measurement	Unit	Water supply	
		Fish 1 rearing	Immunological Testing ²
Total gas saturation	%	100	100
N ₂ saturation	%	99	101-103
O ₂ saturation	%	89	91
pH	umhos	7.7	7.4 ³
Conductivity@ 25°C)	mg/l	244	--
Alkalinity, total (as CaCO ₃)	mg/l	76	--
Hardness, total	mg/l	90	--
Phosphate, ortho (as PO ₄)	mg/l	0.023	--
Solids, dissolved (@ 105°C)	mg/l	180	154
Solids, suspended (@ 105°C)	mg/l	1.2	1
Ammonia, total (as NH ₃ -N)	mg/l	<0.02	<.002
Nitrate (as NO ₃ -N)	mg/l	0.33	.97
Nitrite (as NO ₂ -N)	mg/l	<0.002	<.001
Carbon dioxide	mg/l	3.8	—
Chloride	mg/l	14	22
Cadmium	mg/l	<0.0003	--
Calcium	mg/l	18	17
Cobalt	mg/l	<0.13	<1.0
Copper	mg/l	<0.001	.0025
Iron	mg/l	0.17	.016
Lead	mg/l	0.005	.016
Magnesium	mg/l	11	8
Manganese	mg/l	0.10	.01
Molybdenum	mg/l	<0.16	<.025
Potassium	mg/l	2.8	1.5
Sodium	mg/l	15	8.8
Zinc	mg/l	0.009	.0015
Sulfate	mg/l	19	--
Turbidity	JTU	11	--

¹Abernathy²Oregon State University well³data not available

33with an iodophor (Wescodyne or Argentine)* according to the protocol recommended by Wood (1974). the eggs were then placed in incubator trays (Heath Techna Corp.) supplied with well water. After hatching and yolk absorption, the resulting fry were stocked in a 1200-liter, steel, circular tank furnished with well water at 40 liter/minute. They were then fed ad libitum a proprietary starter formulation (Biodiet - Bioproducts, Warrenton, OR) starter until they reached an average size of about 1.5 grams or larger. Random distribution into each diet group and rearing tank was **performed by** the following procedure. Groups of ten fish were hand counted into separate baskets until a total of 300 fish per basket was reached. Each basket was then assigned to a diet (Each diet used in the study, and its replicate, was written on a separate piece of paper. All the pieces of paper were then placed into a container, mixed, and removed one at a time and assigned to a basket.) Each basket was then assigned to a rearing tank using the same procedure.

Adult New Zealand white female rabbits and BALB/c female mice were maintained by the Laboratory Animal Resource Center on the O.S.U. campus in accordance with the "Guide for the Care and Use of Laboratory Animals" DHEW Publication No. 85.23.

Diets and Feeding. In this year's study, we compared semipurified diets with the Abernathy diet (Table 2), and conducted two preliminary range finding trials with pyridoxine, one range finding trial with folic acid. We also began and formal trials for both pyridoxine and folic acid.

The semipurified diets consisted of 1) a modified Oregon Test Diet (National Science Academy, 1973) and 2) a modified diet supplemented with

¹ listing of any product does not constitute an endorsement

Table 2. Composition of Abernathy Diet used as a vitamin test ration for spring chinook salmon (Oncorhynchus tshawytscha) fingerlings. Immunological study, Abernathy Salmon Culture Technology Center, 1986.

Component	Percent
Herring meal, minimum protein 67.5%	40.57
Dried whey, minimum protein 12%	5.00
Wheat germ meal, minimum protein 23%	5.00
Wheat standard middlings, minimum protein 15%	26.65
Blood meal, spray dried, minimum protein 80%	10.00
Vitamin premix ¹	1.50
Ascorbic acid	0.10
Choline chloride, 60% product	0.58
Trace mineral premix ²	0.10
Herring oil, stabilized with 0.04% BHA-BHT (1:1)	<u>10.50</u>
	100.00

¹ Mg/kg of diet unless otherwise indicated: riboflavin, 53; niacin, 220; folic acid, 12.7; thiamine, 43; biotin, 0.60; B-12, 0.06; vitamin K, 9; inositol, 132; d-pantothenic acid, 106; pyridoxine, 31; vitamin E, 503 IU; vitamin **D₃**, 441 IU; and vitamin A, 6614 IU.

² Mg/kg of diet: zinc, 75.0; manganese, 20.1; copper, 1.54; and iodine, 10.0. Mineral sources were zinc sulfate, manganese sulfate, copper sulfate, and potassium iodate.

6 amino acids (Table 3). The initial pyridoxine range finding study used five levels(15,30,60,120 and 1500 mg/kg) in a variation of the Abernathy Diet. These concentrations represented total values which include the naturally occurring and supplemented vitamins. The natural level of pyridoxine in abernathy diet was 2.5 mg/kg. The complete diet assayed after formulation to verify vitamin concentrations. These analyses determined the following (actual) vitamin concentrations for each of the five groups: 16, 26.7, 60.9, 127.5, and 905.3 mg/kg. The second range finding trial,with pyridoxine, incorporated vitamin levels as shown in Table 4. Table 4 also shows folic acid concentrations used in abernathy diet, used in range finding studies.

The formal vitamin studies incorporated the nominal and actual vitamin concentrations shown in Table 5. All nominal values were quite close to the actual levels found. All vitamin assays were performed by Hazelton Laboratories America, Inc., Madison, Wisconsin.

Meal ingredients for the Abernathy Diet were ground through a hammer mill until they passed entirely through a 20 mesh screen. Components were blended in a paddle mixer and pelletized through a small compaction-type pellet mill without steam conditioning. Sufficient feed for 90 days was prepared at one time and stored at room temperature (22°C) until fed. The dry semi-purified diet ingredients and water were blended in a dough mixer, then frozen (-40°) in airtight containers until used. Fresh feed was prepared at 3 week intervals. Daily allotments of the purified diet were thawed at 4°C and extruded through a ricer to produce strings, which were then cut to the desired pellet length. The particle sizes for the types of diets were chosen according to the guidelines in Table 6. The proximate composition of

Table 3. Composition of two modifications to the Oregon Test Diet formula (National Academy of Sciences, 1973). Immunological study, Abernathy Salmon Culture Technology Center, 1985.

Components	Percentage of Dry Component	
	Modified Oregon Test Diet	Oregon Test Diet w / essential amino acids
Vitamin-free casein	45.9	40.9
Gelatin	8.1	7.2
Dextrin	15.6	15.6
Carboxy methyl cellulose	1.3	1.3
alpha-cellulose	12.129	13.299
alpha-tocopherol (250 IU/g)	0.264	0.264
choline chloride (99%)	0.707	0.707
Mineral mix ^a b	4.0	4.0
Vitamin premix	2.0	2.0
Herring oil	10.0	10.0
L-Arginine	—	0.95
L-Histidine	—	0.38
L-Lysine HCl	—	1.38
L-Methionine	—	1.06
L-Threonine	—	0.77
L-Tryptophan	—	0.26

a,b are identical in composition to those described by the National Academy of Science (1973)

Final diet composition: 35% dry ingredients
65% water

Table 4. Vitamin analysis of preliminary range-finding diets.
Immunological study, Abernathy Salmon Culture Technology
Center, 1986.

Vitamin	mg / kg dry diet	
	Nominal level	Actual level
Pyridoxine	4	5.0
	8	9.0
	15	16.2
	30	31.0
	60	63.0
Folic acid	5	0.7
	6	1.5
	12	5.5
	24	13.0
	48	30.0

Table 5. Vitamin **analysis^a** of formal diets. Immunological study.
Abernathy Salmon Culture Technology Center, 1986.

<u>mg vitamin/kg dry diet</u>				
Diet	Nominal vitamin level	Actual Vitamin Level	n	
		(x+ SE)		
<hr/>				
Abernathy				
Folic acid	6	5.9+0.3		3
	10	10.1+0.4		3
	14	13.1x0.3		3
	18	17.1+1.0		3
	22	20.7+0.6		3
Pyridoxine	15	15.2+0.6		3
	35	39.0+4.0		3
	55	50.2+4.8		3
	75	69.0+1.2		3
	95	88.6+3.7		3
Semi-purified				
Folic acid	2	2.6+0.1		5
	6	6.9+0.2		5
	10	11.5+0.2		5
	14	16.4+0.5		5
	18	20.4+0.5		5
Pyridoxine	5	6.4+0.9		5
	15	17.7+1.0		5
	35	43.3+1.4		5
	55	66.2T3.1		5
	75	83.3+3.9		5

a All vitamin analyses were performed by Hazelton Laboratories America, Inc., Madison, Wisconsin.

Table 6. Guideline for dry feed particle sizes in relation to fish size used in immunological study involving spring chinook salmon (Oncorhynchus tshawytscha) fingerlings at Abernathy Salmon Culture Technology Center, 1985.

Feed Particle Size	Fish Size Range (grams)		
2/64-inch (0.79 mm) granules	0.6	to	1.0
3/64-inch (1.19 mm) granules	1.0	to	2.5
4/64-inch (1.59 mm) granules	2.5	to	5.0
6/64-inch (2.38 mm) granules	5.0	to	6.0
6/64-inch (2.38 mm) pellets ²	6.0	to	10.0
8/64-inch (3.18 mm) pellets	10.0	to	25.0
12/64-inch (4.76 mm) pellets	25.0	to	larger

¹Asymmetrical crumbles.

²Cylinders which have the same length as diameter.

the completed feeds was determined at the Abernathy SCTC using methods described by Horwitz (1980).

Daily feed allotments for all feeds were based upon a uniform weight of dry food per unit weight of live fish computed by the methods of Buterbaugh and Willoughby (1967). Feeding was done by hand with frequencies ranging from hourly, when the fish were small, to four feedings per day for fingerling fish.

Growth and Feed Efficiency. At biweekly intervals during, the rearing phases of tests, each lot of fish was weighed to the nearest gram in a water-filled container. Dead fish were removed from tanks daily and data on their weight and numbers recorded.

Computation of food conversion of specific growth rates (Mahnken et al., 1980), and gross feed efficiencies (Brett et al., 1969) were done as follows:

Specific growth rate, SGR:

$$\text{SGR} = \frac{\log, \text{ of weight at end} - \log, \text{ of weight at start}}{\text{days fed}} \times 100$$

Gross feed efficiency, GFE:

$$\text{GFE} = \frac{\text{total fish weight gain in grams}}{\text{food offered in grams}} \times 100$$

Culture media: Media components were purchased from Whittaker M. A. Bioproducts, Walkersville, MD, unless otherwise noted. Mishell-Dutton

holding medium (HM) consisted of 100 ug/ml gentamicin and 10% fetal calf serum in RPMI 1640 (Gibco). Mishell-Dutton modified RPMI (RPMI MDM) was used for tissue culture and consisted of RPMI supplemented with: non-essential amino acids, sodium pyruvate, L-glutamine, 10% fetal calf serum (hybridoma screened), 100 ug/ml gentamicin, 50 uM 2-mercaptoethanol (MCB, Cincinnati, OH), and the nucleosides, adenosine, uracil, cytosine, and guanine (10 ug/ml, Sigma, St. Louis, MO). The nutritional cocktail was also prepared as previously described (Tittle and Rittenberg, 1978) and fed daily to the cultures as described below.

Mitogens and Antigens: Stock solutions of lipopolysaccharide W (LPS) from *E. coli* 055:B5 (Difco, Detroit, MI) was pasteurized for 30 minutes at 70°C in distilled water. Stock solutions of phytohemagglutinin P (PHA, Sigma) were made up in RPMI MDM and sterilized by filtration through a 0.45 um filter. Trinitrophenylated-LPS (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). All mitogens and antigens were diluted into tissue culture medium at two times the final desired concentration.

Vibrio anguillarum extract. The Vibrio extract was prepared from *V. anguillarum* strain SL-174 which had been formalin killed and stored frozen. Fifty mls thawed packed cells were suspended in ten volumes of 2% saline and placed in a boiling water bath for two hours. Cells were washed three times in 2% saline, centrifuged at 10,000 x g for ten min at 4°C, resuspended in 95% ethanol, and incubated 48 hours at 37°C. The cells were then washed two times in acetone, centrifuging at 3,000 x g for ten minutes and dried to a paste overnight at 37°C. The paste was ground to a fine powder with mortar and pestle and stored at 4°C. The soluble Vibrio extract used for these studies was prepared by boiling the powder

in PBS at ten mg/ml in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1,000 x g to remove particulates and filter sterilized. Protein concentrations were determined by the method of Lowry et al. (1951).

Cell Cultures: For the preliminary, range-finding studies, fish were sacrificed and their spleens and/or anterior kidneys aseptically removed and placed in HM. The organs were transported on ice to the laboratory at OSU for culture preparation. A single cell suspension of each organ was obtained by aspiration through a 1 ml syringe. Organs from six fish were pooled to obtain the required number of cells for culture. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant medium, containing a single cell suspension, was then washed two times in holding medium by centrifugation at 500 x g for 10 minutes at 4°C. The final washed cell pellet was resuspended in RPMI MDM. Lymphocytes were enumerated by the use of a hemocytometer or Coulter counter (Coulter Electronics, Hialeah, FL) adjusted for counting salmonid leukocytes. The cell suspension was then adjusted with RPMI MDM to a concentration of 2×10^7 cells/ml and held on ice until culture. Aliquots of 0.02 ml of the final cell suspension were added to the wells of a 24-well, flat-bottomed, tissue culture plate (Corning, Corning, NY) containing antigen or mitogen. Tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 7% CO₂ at 16°C.

Due to the difficulties in obtaining enough cells, testing a sufficient number of replicate fish per tank, and contamination in our cultures, we modified the assay system for the formal vitamin studies. Space at the Abernathy S.C.T.C. was converted to a tissue culture laboratory, such that cell cultures could be prepared at the Center. Thus, over a two day

period, prepared cultures could be stored, then transported to O.S.U. intact. Also, 96-well tissue culture plates were used for plaque-forming cell assays, cultured anterior kidney lymphocytes from individual fish were used rather than pools of cells from multiple fish, and 12 fish were sampled per tank. The mitogen assay cultures were transported also transported to O.S.U. and harvested there, while the plaque-forming cell assays were harvested at the Abernathy SCTC.

Mitogen Assays. For the mitogen assays, 50 ul of the cell suspension (5×10^5 cells/ml for the preliminary studies and 1×10^6 cells/ml for the final mitogen assays) were placed in individual wells of a 96-well, flat bottom, tissue culture plate with 50 ul of mitogen or culture medium. The plates were then incubated in gas boxes under 7% CO_2 at 17°C . Twenty four hours before harvest each well was pulsed with one uCi of tritiated thymidine (methyl- ^3H , ICN Biomedicals, Irvine, CA) in 50 ul of RPMI MDM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with cocktail (6g PPO, Sigma, 5 mg POPOP, Amersham, Arlington Heights, IL, in one liter toluene, after Etlinger et al., 1976), and counted on a Beckman liquid scintillation counter (EL 3800). Data are reported as mean counts per minute (cpm) +/- standard error, of triplicate cultures, or as stimulation indices (SI) defined as experimental cpm/control cpm.

Plaque-forming Cell Assay. Single cell suspensions of 2×10^7 cells/ml were prepared in RPMI MDM, as described above. For the preliminary assays, 0.2 ml aliquots of cells were added to 0.2 ml of the appropriate dilution of antigen in RPMI MDM or in medium alone, while in the final assays, 0.05 ml of the suspension were cultured with 0.05 ml of antigen or

medium alone. Cultures were fed 50 ul of cocktail for preliminary assays, and 20 ul for final assays, on alternate days until harvest. Cells secreting anti-trinitrophenyl (TNP) antibodies were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). We mixed 100 ul (40 ul for final assays) of the lymphocyte suspension, 25 ul (10 ul) of a 10% suspension of TNP-sheep red blood cells (TNP-SRBC; Rittenberg and Pratt, 1969) in modified barbital buffer (MBB), and 25 ul (10 ul) of steelhead serum, diluted in MBB, in individual wells of a 96-well microtiter plate (Linbro, McLean, VA). The contents of each well was pipetted into a slide chamber, sealed and incubated for 1-2 hours at 16°C. Plaques were then enumerated under low power with the aid of a dissecting microscope.

Disease challenges. Fifty fish from each tank at the Abernathy S.C.T.C. were transferred to the Oregon Fish Disease Laboratory in Corvallis for the disease challenges. The fish were transported in 20 gallon containers with 15 gallons of water aerated with oxygen and cooled with block ice. The fish were allowed to acclimate to their new tanks for two days before disease challenge.

Daily mortalities were counted and kidney smears and/or agar plate checks were performed to verify the presence of the disease organism. Mortalities are reported for each tank as percent mortality over the 12-day period and the mean day to death which is calculated as:

$$\frac{\text{number of mortalities each day X day post challenge}}{\text{total number of mortalities over 12 days}}$$

Vibrio anguillarum. Ten two liter flasks with one liter of

Tryptic Soy Broth (Difco, Detroit, MI) were grown overnight at 25° C with agitation. Water levels were reduced to 20 l/tank and one liter of the broth culture (1.0 O.D. at 540 nm) was added for the immersion challenge. The pathogen was then removed by dilution by resuming normal water flow to the tank.

Aeromonas salmonicida. Twelve 2-liter flasks with 1-liter of Brain Heart Infusion broth (BHI; Difco, Detroit, MI) in each were inoculated with ten ml of a 48 hr A. salmonicida culture grown from a single plate colony. The cultures were gently agitated at 17° C for 24 hrs. Contents of all 12 flasks were pooled and plate counts made on BHI agar plates. For both challenges, cultures contained approximately 10⁹ bacteria/ml as determined by colony counts.

For the immersion challenge, the water level in each tank was drained to approximately two inches above the dorsal fin of the fish and the water flow was terminated. Five hundred mls of the A. salmonicida broth culture was added to each tank. Water flow was resumed 20 min after the bacteria were added.

Renibacterium salmoninarum. Bacteria were grown in KDM-II (Evelyn, 1977) for 7-10 days at 17° C with agitation to 1 O.D. unit. Each tank of fish was anaesthetized with benzocaine and each fish was injected i.p. with 0.05 ml of the unwashed bacterial suspension using a 26 g 1/2" needle and 1-cc syringe.

Differential counts. A drop of blood from a tail cut was placed on a glass slide and smeared with a second slide. The smear was air-dried overnight and fixed for five min in absolute methanol. The smears were stained with Leishman's stain (12 g in 500 ml absolute methanol) for five min, followed by a ten min stain in Giemsa stain (1 g in

66 ml glycerol plus 66 ml absolute methanol). Slides were then rinsed with 1% PBS, then water, and air-dried overnight in the dark. A minimum of 200 cells were enumerated and identified per slide using a 100x oil emersion lens.

ELISA methods for quantifying total immunoglobulin levels and anti-TNP antibodies in fish serum and tissue culture supernatants. A hybridoma cell line producing monoclonal antibodies (McAb, 1-14) to fish Ig was obtained from Dr. G. Warr (Dept. of Microbiology, Univ. of N. Carolina). The cells were injected i.p. into a BALB/c mouse. Approximately two weeks later, the the ascites fluid was aspirated with a needle and syringe, centrifuged, the cells injected into another mouse and the supernatant collected for partial purification of the antibody (1-14). The ascites fluid was cut three times with 50% saturated ammonium sulfate (SAS), then extensively dialyzed against phosphate buffered saline (PBS), sterile filtered, and tested for anti-fish Ig activity.

Biotinylated 1-14 was prepared by dialyzing 3 mg of the SAS cut 1-14 against 0.1 M carbonate buffer and then adding 20 ul of 0.1 M biotinyl-n-hydroxysuccimide ester (Cal-Biochem, La Jolla, CA) in dimethyl formamide. The mixture was agitated at room temperature for one hour, then dialyzed against PBS. The biotinylated 1-14 stock was mixed 1:1 with glycerol and stored in the freezer.

Anti-TNP fish Ig was purified from the serum of coho salmon (Onchorhynchus kisutch) which had been hyperimmunized with TNP-keyhole limpet hemocyanin. The serum was passed over a TNP-Sepharose affinity column, eluted with 3M KSCN, and dialyzed into PBS.

Based on a series of preliminary tests, the following assay system was used to quantify total fish Ig in serum and tissue culture

supernatants. Ninety-six well flat-bottom ELISA plates (Costar, Cambridge, MA) were used for all assays. Tris buffered saline (TBS; 6.07 g Tris base, 8.7 g NaCl, and 0.409 g EDTA-2H₂O in 1 liter of deionized water, pH 8.0) with 0.1% Tween 20 added (TTBS) was used throughout except as indicated. Between each step, the wells were rinsed 4 times with TTBS and then 4 times with TBS.

Wells were coated by overnight incubation with five ug/ml of 1-14 in coating buffer (0.159 g Na₂CO₃ and 0.293 g NaHCO₃ in 100 ml deionized water, pH 9.6) at 17^o C in a covered plate. Wells were then blocked with 1% bovine serum albumin-TBS for one hour at room temperature followed by the addition of dilutions of fish Ig standards and unknowns for three hours. After rinsing, a 1/500 dilution of biotinylated 1-14 in TTBS was added and incubated for two hours, followed by a 1/100 dilution of streptavidin-horseradish peroxidase for 20 min. The substrate (75 ul ABTS, 5 ul H₂O₂, and ten ml 0.2% (w/v) citrate buffer, pH 4.0) was then added. Optical densities were read at ten min intervals at wavelength = 405 on a Biotek EL310 ELISA reader (Burlington, VT, 05401).

For quantification of TNP-specific antibodies, the same procedure was followed, using the same reagents, except the plates were coated with 0.5 ug/ml TNP-BSA in coating buffer and the standards and unknowns were left on the plates overnight at 17^o C.

RESULTS AND DISCUSSION

I. Comparison of Diet Formulations - Abernathy and Semi-purified.

Growth and Feed Efficiencies. In this phase of the study, the growth rate and feed efficiency of the diets to be used were tested. The specific growth rates demonstrated significant differences (Table 7). The essential amino acid supplemented Oregon Test Diet (OTD) possessed the highest growth rate followed by the unsupplemented OTD, and Abernathy Diet. The gross feed efficiency was greatest for the amino acid supplemented OTD followed by unsupplemented OTD, and the Abernathy diet.

The specific growth rate of the modified Oregon Test Diets (1.220 - 1.230), supplemented with essential amino acids or glutamic acid and glutamic hydrochloride, approached the specific growth rates found with the Abernathy diets (1.240 - 1.264). In comparing the gross feed efficiency, the Oregon Test Diets produced higher values (74.02 - 74.12) than found with the Abernathy diets (64.88 - 66.05). Since this was an exploratory study, no statistical inference was made. Our results indicate that there was no reason to supplement the Oregon Test Diet.

II. First preliminary range-finding study.

Growth and Feed Efficiencies. The results indicate no significant difference in the specific growth rate and gross feed efficiency, among the five levels of pyridoxine (15, 30, 60, 120 and 1500 mg/kg diet). Based on these results, the levels of pyridoxine above 15 mg/kg do not appear to

Table 7. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fingerlings fed modified Oregon Test Diet (National Academy of Sciences, 1973), Oregon Test Diet supplemented with essential amino acids, and Abernathy diet for 84 days. Immunological study, Abernathy Salmon Culture Technology Center, 1985.

Diet	Specific ^{A,C} Growth Rate	Gross ^{B,C} Feed Efficiency
Modified Oregon Test Diet	0.538±0.002 ^a	55.65±0.51 ^d
Oregon Test Diet with essential amino acids	0.593±0.002 ^b	61.76±0.83 ^e
Abernathy Diet	0.471±0.008 ^c	45.60±1.35 ^f

A Specific growth =
$$\frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

B Gross Feed Efficiency =
$$\frac{\text{total fish weight gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

C Values are x±SE, n =2, Values with different letter designations are significantly different (p< 0.05)

restrict the growth rate (Table 8).

Plaque-forming cell responses. The ability of animals from the various treatment groups to produce specific antibody to trinitrophenylated-lipopolysaccharide (TNP-LPS) was assessed by the use of the *in vitro* production of plaque forming cells (PFC). Thus, after the fish are raised on the various diets, the immune organs (anterior kidney and spleen) are removed and cultured with antigen. The cells are then harvested after 9 days and assessed for the number of PFC. This is an extremely sensitive measure of antibody production, and was used along with the mitogenic assays to detect possible changes in the immune system which may not be detected under the conditions of an experimental disease challenge.

Figure 1 depicts the response of splenic lymphocytes from animals raised on various pyridoxine diets. The figure demonstrates that there was a significant and positive effect due to the increase of the pyridoxine concentration over that required by the N.R.C. (15 mg/kg diet). Thus a four-fold (60 mg/kg diet) increase in the concentration of pyridoxine resulted in a two-fold increase in antibody production (PFC).

Another point to be made from this data is the effect of extremely high concentrations of pyridoxine on PFC formation. In the case of 120 mg/kg diet and 1500 mg/kg diet supplements to the diet, the PFC response was reduced to that seen with the minimal requirement.

Response to *Vibrio anguillarum* extract. The *in vitro* responsiveness to the salmonid pathogen, *Vibrio anguillarum*, was assessed by the stimulation of lymphocytic proliferation by soluble *V. anguillarum* extract. The proliferation of the lymphocytes was determined by the uptake of tritiated

Table 8. Specific growth rate and gross feed efficiency of spring chinook (Oncorhynchus tshawytscha) fingerlings fed different pyridoxine concentrations in the Abernathy Diet. Immunological study, Abernathy Salmon Culture Technology Center, 1985.

Abernathy Diet Nominal mg pyridoxine/kg Diet ^D	Specific ^{A,C} Growth Rate	Gross ^{B,C} Feed Efficiency
15	1.264+0.008	66.05+0.38
30	1.252+0.004	65.72+0.57
60	1.240+0.008	65.56+1.05
120	1.248+0.000	64.88+0.23
1500	1.252+0.020	65.8+2.03

$$A \text{ Specific growth} = \frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

$$B \text{ Gross Feed Efficiency} = \frac{\text{total fish weight gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

^C Values are x+SE, n =2.

^D Total pyridoxine level, includes both the basal level found in the natural ingredients and supplemented pyridoxine.

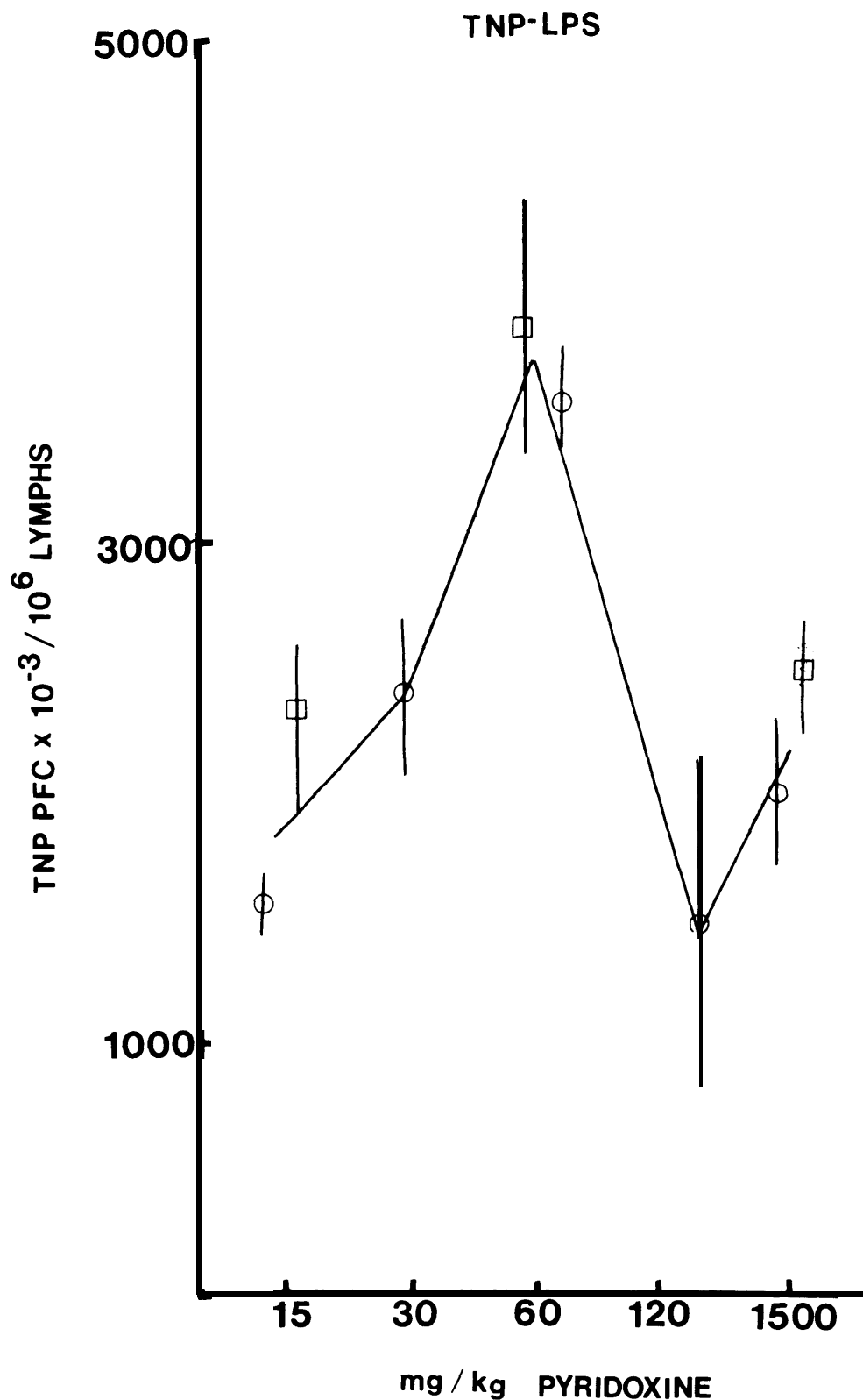


Figure 1. Plaque-forming response for the first preliminary pyridoxine range-finding study. Plaque-forming cell response to trinitrophenylated-lipopolysaccharide is presented. \square represents the average values for each replicate tank. \circ represents the average of triplicate pools of splenic lymphocytes from six animals. The bars represent the standard error of the mean.

thymidine (Fig 2). As was seen with the antibody forming response, the mid-range values (30 and 60 mg/kg) of pyridoxine were found to produce the greatest degree of responsiveness. The replicate values again were quite close and had similar variances. The background responses were less than 1% of the stimulated counts per minute (cpm) in all cases.

As with the antibody responses, the higher doses of pyridoxine (120 and 15000 mg/kg) demonstrated a marked decrease in the amount of tritiated thymidine incorporation. The 1500 mg/kg level actually produced a response that was less than that seen with the lowest level of vitamin. This may indicate some pyridoxine toxicity to immune tissues at extremely high concentrations (i.e. 100-fold over the recommended level).

These results are also presented in the form of stimulation indices (Fig 3), This form of data analysis normalizes the data by dividing each stimulated culture by the corresponding background count. This form of analysis also demonstrates the mid-range vitamin concentrations produces enhanced responsiveness. However, in this case, 30, 60, and 120 mg/kg possess equal responsiveness to the vibrio extract. Evaluation of both expressions of the data leads to the conclusion that the mid-range values for pyridoxine supplementation give the greatest degree of enhancement.

Response to Escherichia coli lipopolysaccharide (LPS). Further delineation of the effects of pyridoxine supplementation were examined by the use of the B cell mitogen, E. coli LPS. Measurement of the total incorporation of tritiated thymidine (Fig 4) reveals that all pyridoxine concentrations except the 60 mg/kg concentration produced approximately control levels of mitogen responsiveness. Thus, once again, mid-range values of pyridoxine give the highest degree of responsiveness, and higher levels result

VIBRIO

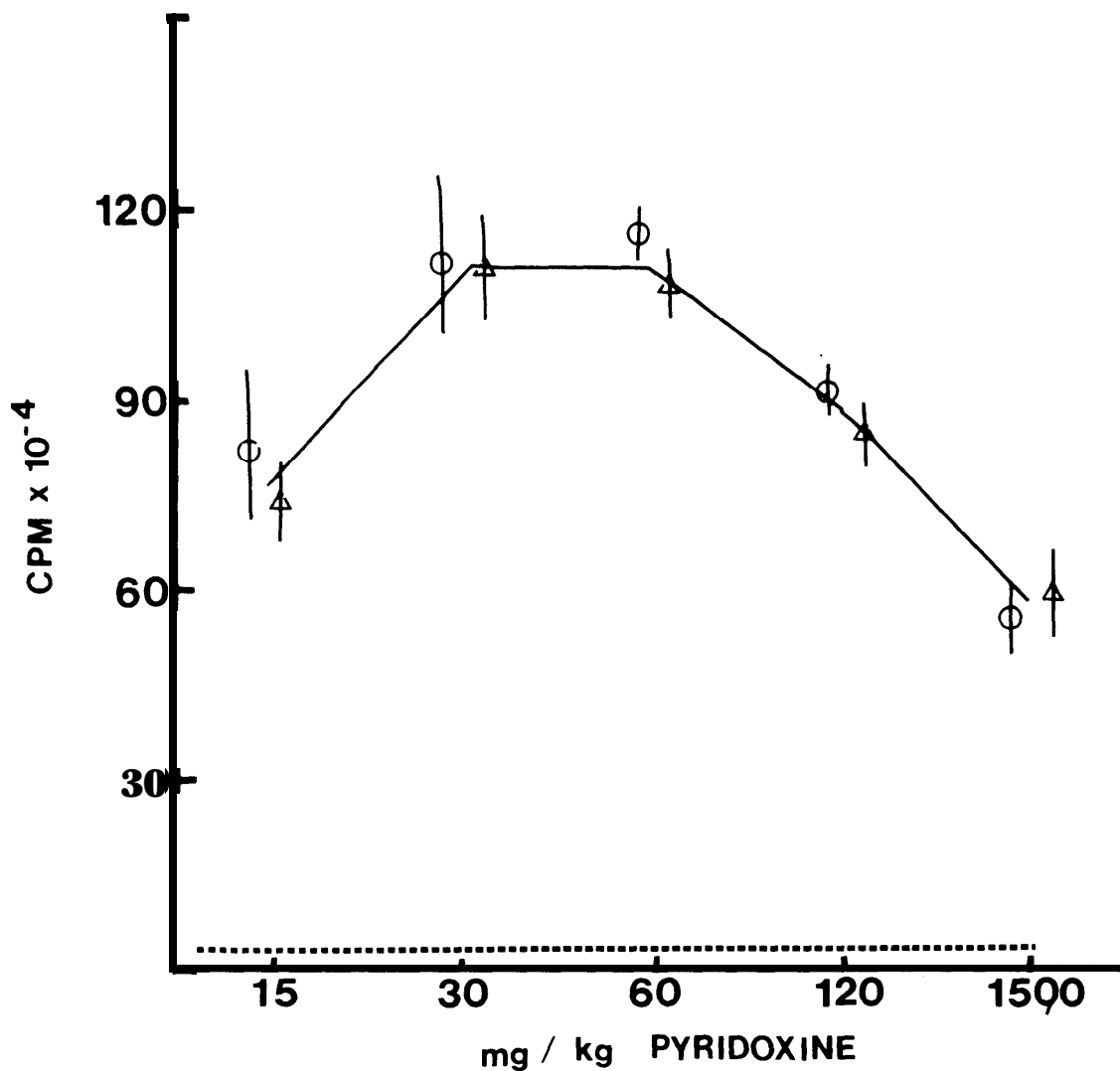


Figure 2. Vibrio anguillarum mitogenesis response for the first preliminary pyridoxine range-finding study.
Mitogenic response to Vibrio anguillarum extract.
 O Δ represent the average values for each replicate tank,
 Each symbol represents the average of triplicate pools
 of splenic lymphocytes from six animals. The dotted line
 represents the counts per minute of unstimulated cultures,
 The bars represent the standard error of the mean.

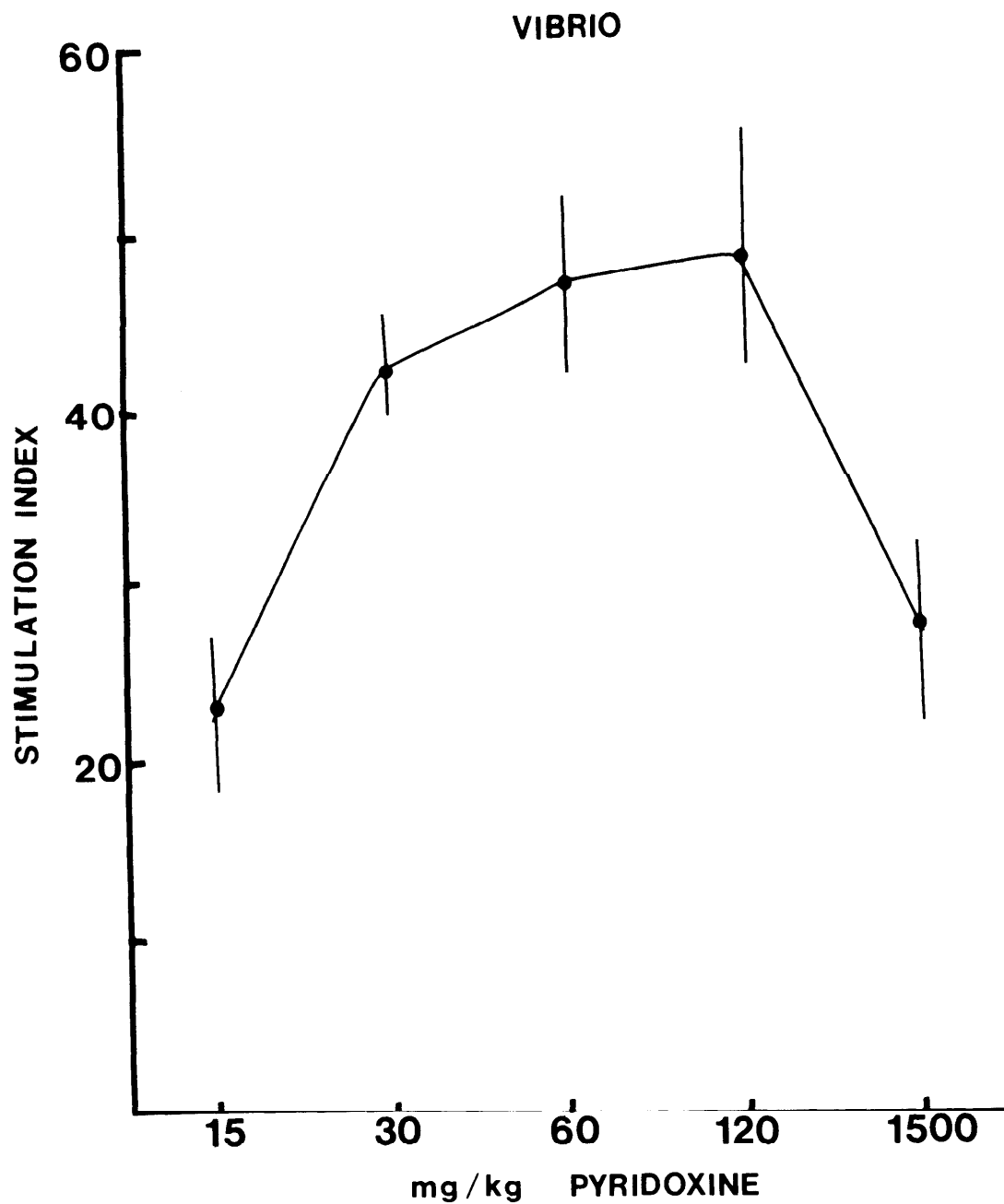


Figure 3. Vibrio anguillarum mitogenesis response for the first preliminary pyridoxine range-finding study. Mitogenic response to Vibrio anguillarum extract expressed as stimulation indices. | represents the average values for each replicate tank. Each symbol represents the average of triplicate pools of splenic lymphocytes from six animals. The bars represent the standard error of the mean.

LIPOPOLYSACCHARIDE

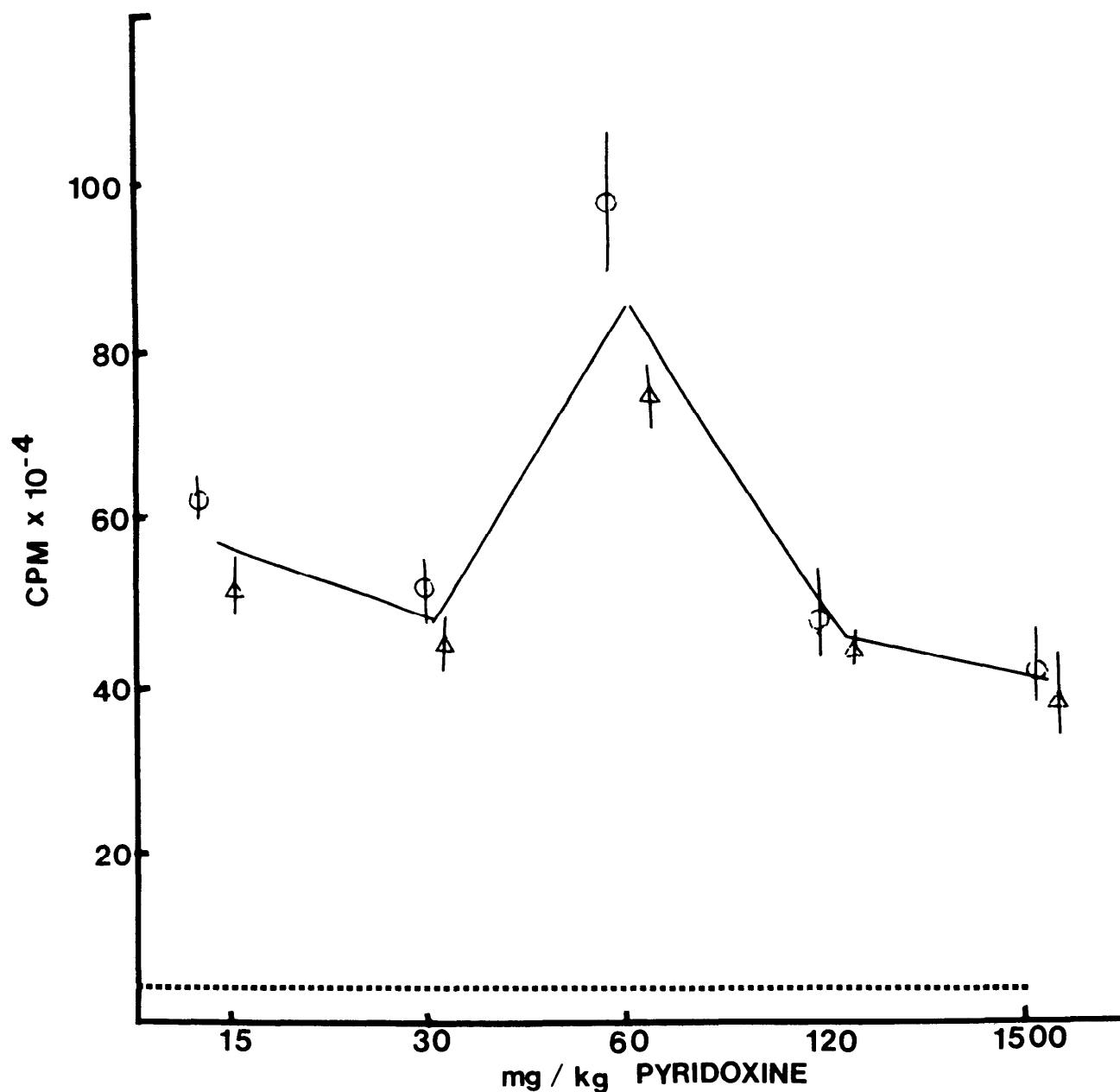


Figure 4, *E. coli* mitogenesis response for the first preliminary pyridoxine range-finding study. Mitogenic response to *Escherichia coli* lipopolysaccharide expressed in counts per minute. Δ represent the average values for each replicate tank. Each symbol represents the average of triplicate pools of splenic lymphocytes from six animals. The dotted line represents the counts per minute of unstimulated cultures. The bars represent the standard error of the mean.

in lower, or control levels of responsiveness.

Response to Phytohemagglutinin. The response to phytohemagglutinin (PHA), which is commonly thought to be a T cell mitogen, expressed no differences at any of the concentrations of pyridoxine (Fig 5). It must be noted, however, that the responses were quite low when compared to those elicited by Vibrio or LPS. Therefore, it may be difficult to discern any T cell differences in responsiveness using PHA

Other Assays Serum immunoglobulin levels were determined for individual fish from each group. Animals from all treatments possessed the same level of immunoglobulin (2.5 mg/ml serum). Treatments with low levels of serum immunoglobulin would be expected if certain diets were deficient, however, diets that are equivalent or superior in their nutritional value may not demonstrate heightened immunoglobulin levels above that for a normal animal.

Phagocytic indices were determined by the ingestion, by phagocytes, of latex beads. Cells from all animals demonstrated the same degree of phagocytosis, as measured by this assay. Future work is planned to examine whether the phagocytic cells possess variations in their functional or bacteriocidal processes by the use of the chemiluminescence assay. Thus, we should be able to assess the respiratory burst that occurs after the ingestion of bacterial cells.

All differential staining of blood cells revealed no significant differences in the number of lymphocytes (93%), granulocytes (3.0%), polymorphonuclear cells (2%), monocyte-macrophages (1.0%).

III. Second preliminary range-finding study.

PHYTOHEMAGGLUTINININ

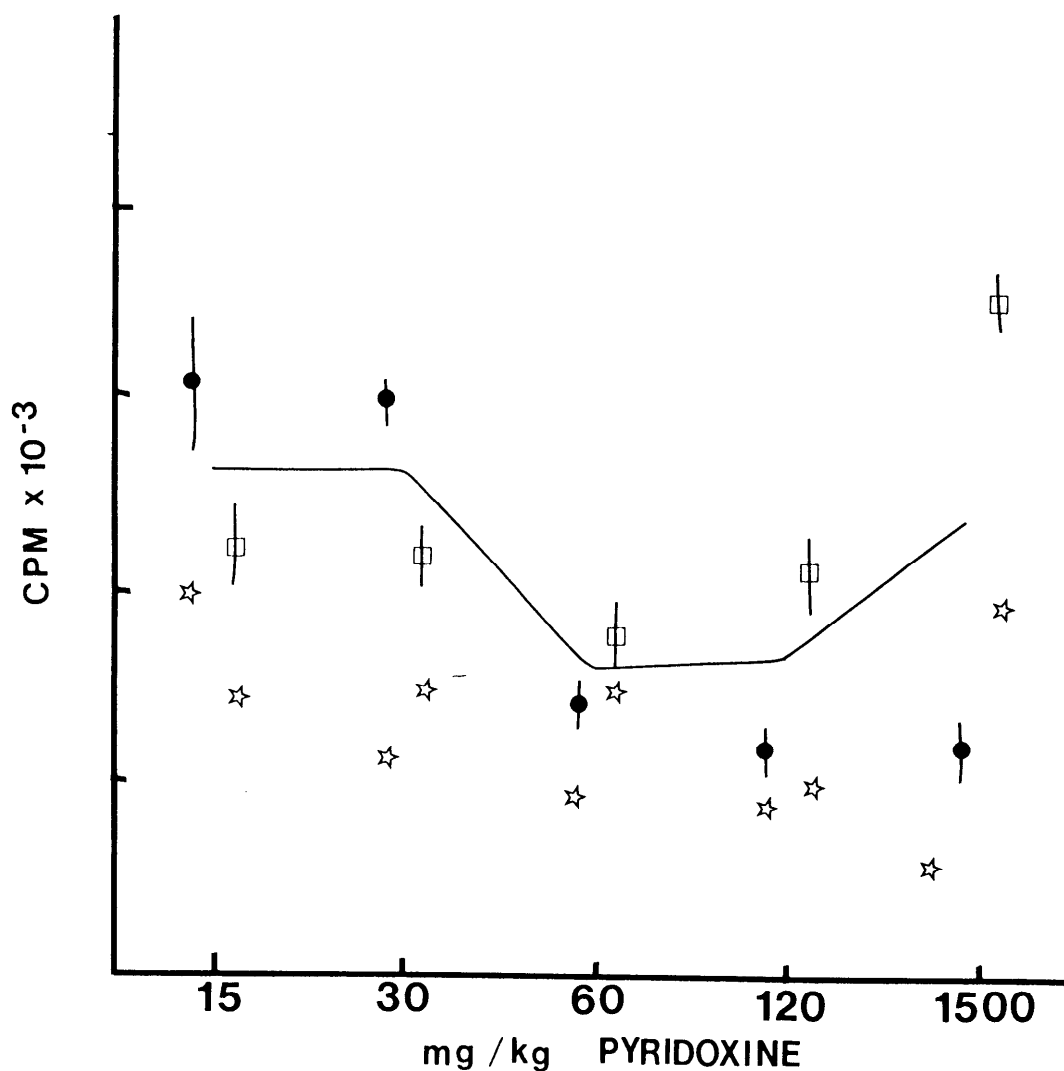


Figure 5. Phytohemagglutinin mitogenesis response for the first preliminary pyridoxine range-finding study. Mitogenic response to phytohemagglutinin expressed as count per minute. \square \star \bullet represent the average values for each replicate tank of stimulated cultures, and represents the values for unstimulated cultures. Each symbol represents the average of triplicate pools of splenic lymphocytes from six animals. The bars represent the standard error of the mean.

Immunological Studies. Technical problems with in vitro immunological tests arose during the performance of the second preliminary trial with pyridoxine, such that the data were considered unreliable. Therefore only the results obtained with folic acid will be discussed.

Growth and feed efficiencies. The second preliminary range-finding trial produced no differences in the specific growth rates or gross feed efficiencies for all levels of pyridoxine (4 - 60 mg / kg) and folic acid (5 - 48 mg / kg) in Abernathy diet (Table 9).

Plaque-forming cell responses. The plaque-forming assay revealed distinctly different anti-TNP antibody responses for the different vitamin concentrations (Fig 6). The mid-range concentrations (1.5 and 5.5 mg / kg) produced lower responses than the lowest concentration (0.7 mg / kg) or the highest concentration (13 and 30 mg / kg).

Response to *Vibrio anguillarum* extract. The proliferative response to the vibrio extract demonstrated a distinctly different effect than that seen with anti-TNP response (Fig 7), with the mid-range concentration of 5.5 mg / kg folic acid giving the highest degree of responsiveness. The other concentrations, both higher and lower, generated approximately half the stimulation of the 5.5 mg /kg concentration.

Disease challenge with *Vibrio anguillarum*. Although our formal trials specify the use of *Aeromonas salmonicida* and *Renibacterium salmoninarum* for the disease challenges, in this preliminary range-finding trial *Vibrio anguillarum* was used. Challenge with *V. anguillarum* revealed

Table 9. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fingerlings fed different dietary concentrations of pyridoxine and folic acid in the Abernathy diet for 112 days. Immunological study, Abernathy Salmon Culture Technology Center, 1985.

Vitamin	Nominal vitamin level mg vitamin/kg dry diet	Specific ^{A,C} Growth Rate	Gross ^{B,C} Feed Efficiency
Pyridoxine	4	0.637±0.009 ^a	59.66±0.50 ^b
	8	0.554±0.018 ^a	48.94±1.55 ^b
	15	0.577±0.039 ^a	51.04±3.90 ^b
	30	0.572±0.033 ^a	51.78±2.45 ^b
	60	0.622±0.020 ^a	56.71±1.36 ^b
Folic acid	5	0.548±0.015 ^c	51.61±0.33 ^d
	6	0.597±0.017 ^c	56.24±1.13 ^d
	12	0.584±0.000 ^c	54.97±0.07 ^d
	24	0.582±0.026 ^c	54.88±3.50 ^d
	48	0.536±0.008 ^c	49.76±0.78 ^d

$$^A \text{ Specific growth} = \frac{\log \text{ of weight at end} - \log \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^B \text{ Gross Feed Efficiency} = \frac{\text{total fish weight gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

^C Values are $\bar{x} \pm \text{SE}$, n =2. Values with different letter designations are significantly different (p< 0.05)

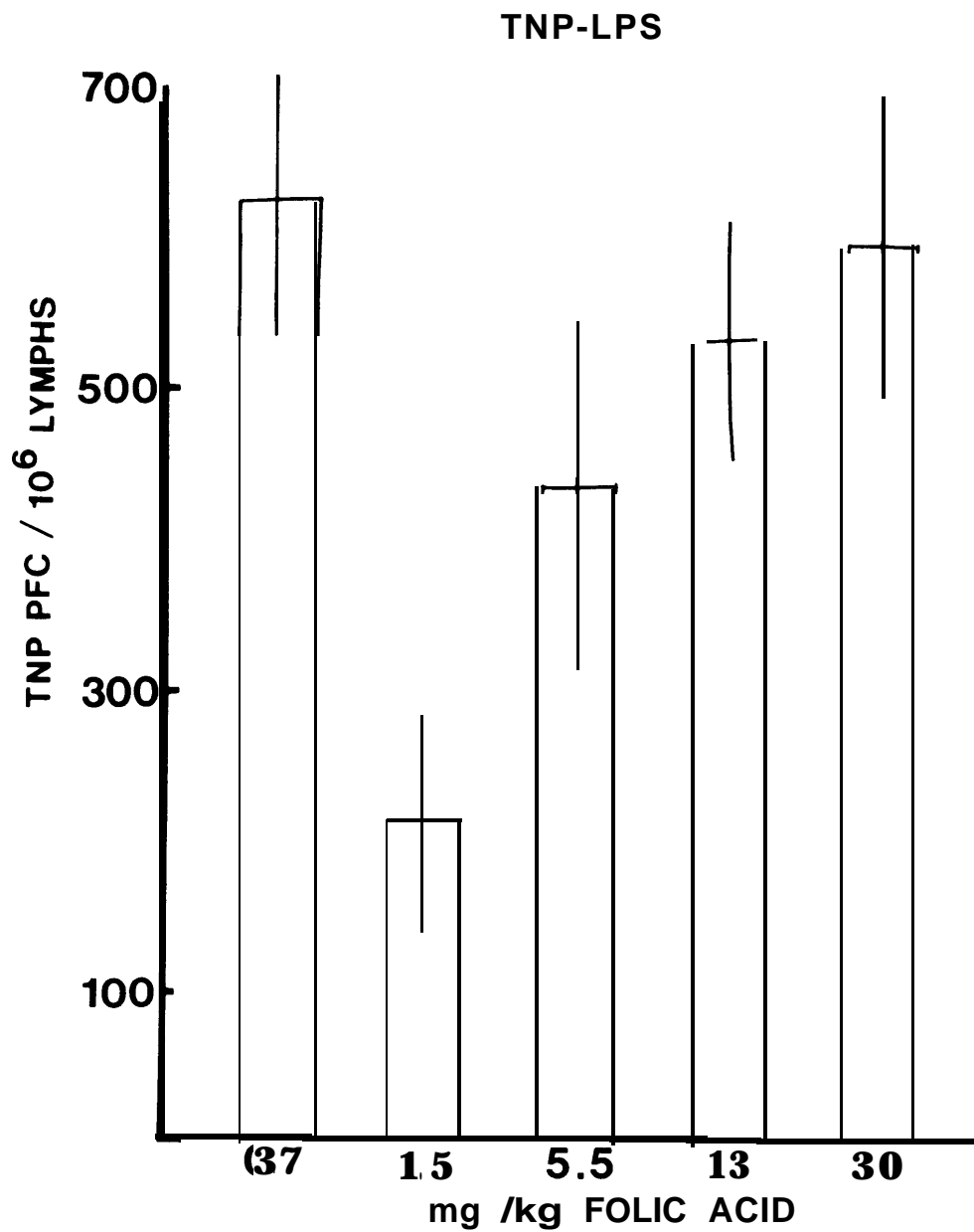


Figure 6. Plaque-forming response for the second preliminary folic acid range-finding study. Plaque-forming cell response of splenocytes to trinitrophenylated-lipopolysaccharide is presented. The columns represent the average values for both replicate tanks. The bars represent the standard error of the mean.

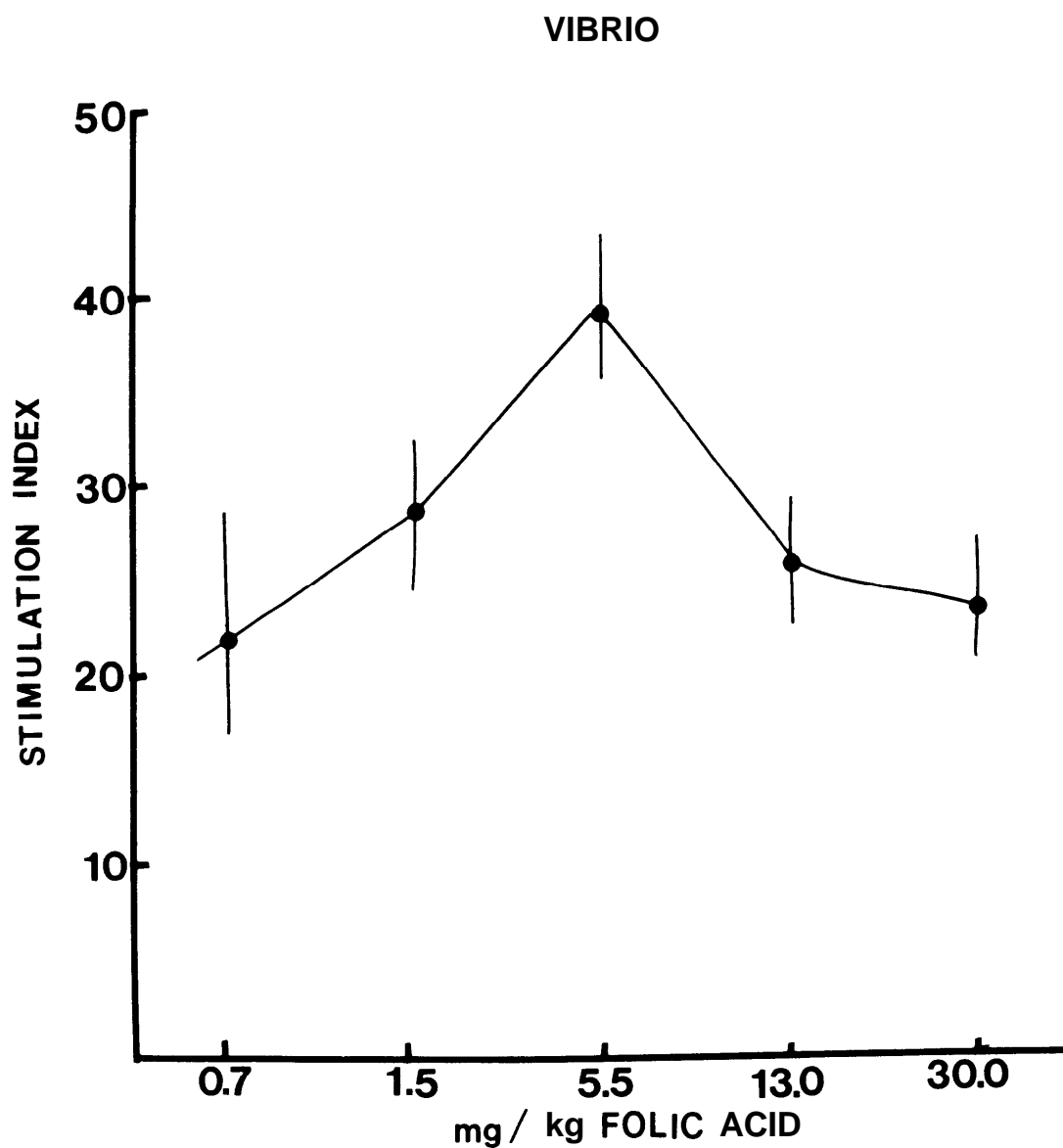


Figure 7. Vibrio anguillarum mitogenesis response for the second preliminary folic acid range-finding study. Mitogenic response to Vibrio anguillarum extract expressed as stimulation indices. \bar{x} represents the average values for each replicate tank. Each symbol represents the average of triplicate pools of splenic lymphocytes from six animals. The bars represent the standard error of the mean.

no statistical differences in the mortalities for the four highest folic acid concentrations (Table 10). The lowest level of folic acid produced approximately 35 per cent fewer mortalities, unfortunately the value of 53 % mortality is based on one tank. It is of interest though, that the 0.7 mg / kg concentration of folic acid also possessed the highest plaque-forming cell response.

IV. Formal trials for pyridoxine and folic acid. The formal diet trials are currently being conducted as this annual report is being written. Therefore, the data are incomplete and a number of assays have yet to be performed or completed. These include: the Vibrio extract mitogenesis, the balance of the plaque-forming assays, the Renibacterium salmoninarum challenges, the estimates of phagocytic activity, and the differential staining of blood smears. All experiments are not entirely complete and thus the final statistical analysis will await the acquisition of all the data (approximately July 30, 1986).

Growth and feed efficiencies. The results indicate that the various vitamin concentrations of pyridoxine and folic acid, whether in Abernathy or in a semi-purified diet, do not significantly alter the growth rate or feed efficiency (Tables 11 and 12), except for the highest concentration of folic acid in the semi-purified diet (Table 11). At this concentration (18 mg/kg) a significant decrease in the specific growth rate is seen, although the gross feed efficiency was not significantly lower at this point. It may be of interest to note, however, that the gross feed efficiency is decreasing steadily as the folic acid concentration is increasing. In all cases, the Abernathy diets appear to give a slightly higher specific growth rate and gross feed efficiency than do the semi-purified diets.

Table 10, Vibrio anguillarum challenge of fish fed various levels of folic acid in the preliminary range finding trial. Folic acid was incorporated in Abernathy dry diet. Thirty fish were tested for each vitamin concentration.

Folic Acid - Actual level (mg/kg dry diet)	Mortality (% of 30 animals)
0.7	-* 53
1.5	83, 86
5.5	90, 96
13.0	73, 80
30.0	90, 83

* Tank of animals lost due to mechanical difficulties.

Table 11. Specific growth rate and gross feed efficiency of spring chinook (Oncorhynchus tshawytscha) fingerlings fed two diets containing different dietary concentrations of folic acid for 111 days. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Nominal vitamin level mg folic acid/kg dry diet	Specific ^{A,C} Growth Rate	Gross ^{B,C} Feed Efficiency
Abernathy	6	1.844±0.015 ^a	81.29±1.64 ^d
	10	1.876±0.025 ^a	83.00±2.16 ^d
	14	1.832±0.010 ^a	80.51±0.97 ^d
	18	1.822±0.009 ^a	80.55±0.92 ^d
	22	1.844±0.006 ^a	80.83±0.42 ^d
Semi-purified	2	1.690±0.005 ^b	71.85±0.58 ^e
	6	1.704±0.005 ^b	71.52±0.92 ^e
	10	1.672±0.008 ^b	70.46±0.20 ^e
	14	1.668±0.000 ^b	69.86±0.05 ^e
	18	1.642±0.002 ^c	68.54±1.27 ^e

$$^A \text{ Specific growth} = \frac{\log \text{ of weight at end} - \log \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^B \text{ Gross Feed Efficiency} = \frac{\text{total fish weight gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

^C Values are $\bar{x} \pm \text{SE}$, n =2, Values with different letter designations are significantly different (p< 0.05)

Table 12. Specific growth rate and gross feed efficiency of spring chinook (*Oncorhynchus tshawytscha*) fingerlings fed two diets containing different dietary concentrations of pyridoxine for 125 days. Immunological study. Abernathy Salmon Culture Technology Center, 1986.

Diet	Nominal vitamin level mg pyridoxine/kg dry diet	Specific ^{A,C} Growth Rate	Gross ^{B,C} Feed Efficiency
Abernathy	15	1.729±0.018 ^a	69.67±1.76 ^c
	35	1.721±0.004 ^a	67.08±1.99 ^c
	55	1.752±0.007 ^a	71.44±0.98 ^c
	75	1.742±0.020 ^a	70.30±1.71 ^c
	95	1.727±0.004 ^a	69.34±0.15 ^c
Semi-purified	5	1.632±0.001 ^b	65.40±0.21 ^d
	15	1.577±0.007 ^b	60.78±0.30 ^d
	35	1.629±0.011 ^b	63.22±2.37 ^d
	55	1.605±0.010 ^b	59.14±0.79 ^d
	75	1.599±0.001 ^b	62.29±0.97 ^d

$$^A \text{ Specific growth} = \frac{\log_e \text{ of weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$$

$$^B \text{ Gross Feed Efficiency} = \frac{\text{total fish weight gain in grams}}{\text{total food (dry weight) offered in grams}} \times 100$$

^C Values are x±SE, n =2. Values with different letter designations are significantly different (p< 0.05)

Plaque-forming cell responses. The anti-TNP plaque-forming cell responses generated in anterior kidney lymphocytes revealed that mid-range concentration of 35 mg pyridoxine / kg of semi-purified diet produced the highest degree of responsiveness (Table 13). It is of interest to note that the highest concentration (75 mg/kg) demonstrated a lower value than that seen with the minimal requirement (15 mg/kg).

The PFC responses for pyridoxine in Abernathy diet revealed a distinctly different phenomenon (Table 14). In this case, the 15 mg / kg concentration demonstrated the highest degree of stimulation, and , once again, the highest concentration (95 mg/kg) gave the lowest amount of stimulation.

Response to E. coli lipopolysaccharide. Examination of the stimulation indices generated by lipopolysaccharide reveals a steady increase in values with increasing pyridoxine in the semi-purified diet (Table 15). The highest concentration (75 mg/kg) produced the highest stimulation index (25.0±0.7). If the counts per minute (Table 15) are examined, there does not appear to be a direct correlation. This finding suggests that the background level of counts incorporated may have some relation to the amount of counts capable of being incorporated upon mitogenic stimulation. When the various levels of pyridoxine are incorporated in Abernathy diet, a mid-range concentration seems to give optimal stimulation (Table 16). This appears to be the case for both data expressed as stimulation indices or counts per minute. Also, in both cases, the highest level of pyridoxine produces sub-optimal levels of stimulation.

Disease challenges with Aeromonas salmonicida. Tables 17 and 18 demonstrate the mortalities for fish challenged after being raised on various pyridoxine levels in semi-purified and Abernathy diets, respectively.

Table 13. In vitro antibody forming responses to TNP-lipopolysaccharide (0.5 us/ml) of anterior kidney lymphocytes from fish fed various pyridoxine levels in the semi-purified diet. Responses are presented as mean numbers of plaque forming cells (PFC)/culture wells + one standard error, for each replicate tank and for the two tanks combined. n = the number of fish sampled.

Pyridoxine (mg / kg)	Tank	n	PFC / Culture	
			Replicate Tank Values	Mean + SE of Replicates
5	8A	12	3,140 + 710	3,200 + 60
5	8B	12	3,260 + 440	
15	10A	12	3,670 + 800	3,505 + 165
15	10B	12	3,340 + 550	
35	7A	12	3,340 + 510	3,870 + 480
35	7B	12	4,800 + 550	
55	6A	12	3,290 + 590	3,675 + 385
55	6B	12	4,060 + 770	
75	9A	12	2,620 + 260	2,895 + 275
75	9B	12	3,170 + 350	

Table 14. In vitro antibody forming responses to TNP-lipopolysaccharide (0.5 ug/ml) of anterior kidney lymphocytes from fish fed various pyridoxine levels in Abernathy diet. Responses are presented as mean numbers of plaque forming cells (PFC)/culture wells + one standard error, for each replicate tank and for the two tanks combined. n = the number of fish sampled.

Pyridoxine (mg / Kg)	Tank	n	PFC / Culture	
			Replicate Tank Values	Mean + SE of Replicates
15	2A	10	2,160 + 660	2,090 + 70
15	2B	10	2,020 + 470	
35	3A	7	1,820 + 410	1,840 + 20
35	3B	3	1,860 + 730	
55	5A	6	1,460 + 330	1,200 + 260
55	5B	10	940 + 230	
75	4A	6	1,530 + 540	1,505 + 25
75	4B	8	1,480 + 500	
95	1A	8	1,030 + 470	1,040 + 10
95	1B	8	1,050 + 550	

Table 15. Mitogenic responses to E coli lipopolysaccharide (100 ug/ml) of anterior kidney lymphocytes from fish fed various pyridoxine levels in the semi-purified diet. Responses are presented as mean stimulation index (SI) and net counts per minute (cpm) + one standard error for each replicate tank and the two tanks combined. n = number of fish tested.

Pyridoxine (mg / kg)	Tank	n	Stimulation Index		cpm	
			Replicate Values	Mean of Replicates	Replicate Values	Mean of Replicates
5	8A	12	14.1+2.2	15.9+1.8	1716123253	22175~5014
5	8B	12	17.6+2.3		27189+4329	
15	10A	12	15.9+2.0	17.7+1.8	24215~3458	28259+4043
15	10B	12	19.4+2.7		32302~3920	
35	7A	12	13.6+2.0	17.2+3.6	2655124313	2848721935
35	7B	12	20.8+2.8		30422~3508	
55	6A	11	19.4+1.7	18.3+1.1	21573~3221	23592+2019
55	6B	12	17.1+3.0		25611~3680	
75	9A	12	25.7+3.1	25.0+0.7	3527323659	2836626907
75	9B	12	24.3+2.7		21459+3172	

Table 16. Mitogenic responses to *E. coli* lipopolysaccharide (100 ug/ml) of anterior kidney lymphocytes from fish fed various pyridoxine levels in Abernathy Diet. Responses are presented as mean stimulation index (SI) and net counts per minute (cpm) + one standard error for each replicate tank and the two tanks combined. n = number of fish tested.

Pyridoxine (mg / kg)	Tank	n	Stimulation Index		cpm	
			Replicate Values	Mean of Replicates	Replicate Values	Mean of Replicates
15	2A	10	11.0+2.1	10.1+.38	22297+4991	219892307
15	2B	10	9.3+1.7		21681+3024	
35	3A	7	11.9+1.5	12.8+0.4	22531~3183	23127+595
35	3B	3	13.7+1.3		23722+3183	
55	5A	6	15.7+4.7	12.9+1.2	23887~6223	21713+2174
55	5B	10	10.2+1.3		19540+2774	
75	4A	6	10.3+2.4	11.2+0.4	2648926763	25257+1232
75	4B	8	12.2+1.1		24025+3557	
95	1A	8	8.5+_3.0	8.8+0.1	950923129	12439+2930
95	1B	8	9.1+2.8		15370+3748	

Table 17. Per cent mortality and mean day to death for fish fed various levels of pyridoxine in the semi-purified diet upon challenge with Aeromonas salmonicida. Fifty fish were challenged per tank.

Pyridoxine (mg / kg)	Tank	% Mortality		Mean day to Death	
		Replicate Values	Tank Mean of Replicates	Replicate Values	Tank Mean of Replicates
5	8A	88	86+2.0	8.1	7.85+0.25
5	8A	84		7.6	
15	10A	84	83+1.0	7.5	7.55+0.05
15	10B	82		7.6	
35	7A	88	92+23.9	6.8	6.8+0
35	7B	96		6.8	
55	6A	78	81+3.0	7.8	8.0+0.2
55	6B	84		8.2	
75	9A	94	87+7.0	7.7	7.95+0.02
75	9A	80		8.2	

Table 18. Per cent mortality and mean day to death for fish fed various levels of pyridoxine in Abernathy Diet upon challenge with Aeromonas salmonicida. Fifty fish were challenged per tank.

Pyridoxine (mg / kg)	Tank	% Mortality		Mean day to Death	
		Replicate Values	Tank Mean of Replicates	Replicate Values	Tank Mean of Replicates
15	2A	50	51+1	9.3	9.55+0.25
15	2A	52		9.3	
35	3A	64	55+9	8.5	9.3+0.8
35	3B	46		10.1	
55	5A	40	34+6	9.5	9.8+0.3
55	5B	28		10.1	
75	4A	38	35+3	5.9	9.35+0.45
75	4B	32		9.8	
95	1A	38	40+2	9.3	9.3+0
95	1B	42		9.3	

Challenge of the semi-purified diet groups revealed no differences in either the per cent mortality nor in the mean day to death (Table 17). The Abernathy diet groups (Table 18) show that the mid-range concentrations appear to have lower levels of mortality (55 and 75 mg/kg). No significant differences can be seen in the mean day to death.

Another point of interest was the accelerated mortality (reduced mean time to death) and greater cumulative mortality in the semi-purified diet group as compared to the Abernathy diet groups (Tables 17, 18 and Fig 8).

The folic acid concentrations demonstrated no statistical differences in the mortalities or the mean times to death, when incorporated in either the semi-purified (Table 19) or in the Abernathy diet.

Table 19. Per cent mortality and mean day to death for fish fed various levels of folic acid in the semi-purified diet upon challenge with Aeromonas salmonicida. Fifty fish were challenged per tank.

		% Mortality		Mean day to Death	
Folic Acid Tank (mg / kg)		Replicate Tank Values	Mean of Replicates	Replicate Tank Values	Mean of Replicates
2	17A	84	8925.0	7.6	7.80+_0.30
2	17B	94		7.0	
6	18A	66	80+_14.0	7.5	7.05+0.45
6	18B	94		6.6	
10	16A	88	82+6.0	6.6	7.35+0.75
10	16B	76		8.1	
14	20A	62	63+1.0	7.5	7.55+0.05
14	20B	64		7.6	
18	19A	58	72+14.0	8.1	7.40+_0.70
18	19B	86		6.7	

Table 20. Per cent mortality and mean day to death for fish fed various levels of folic acid in Abernathy Diet upon challenge with Aeromonas salmonicida. Fifty fish were challenged per tank.

Folic Acid Tank (mg 1 kg)		% Mortality		Mean day to Death	
		Replicate Values	Tank Mean of Replicates	Replicate Values	Tank Mean of Replicates
6	11A	a		a	
6	11B	96		6.4	
10	15A	52	70+ _{18.0}	8.1	7.55+ _{0.55}
10	15B	88		7.0	
14	12A	80	81+ _{1.0}	7.4	7.5+ _{0.1}
14	12B	82		7.6	
18	13A	76	71 _{25.0}	7.3	7.1+ _{0.2}
18	13B	66		6.9	
22	14A	48	58+ _{10.0}	8.5	7.85+ _{0.65}
22	14B	68		7.2	

SUMMARY AND CONCLUSIONS

The primary goals in the second year of this project were to perform preliminary range-finding trials on pyridoxine and folic acid supplementation of the Abernathy practical ration. Following the analysis of the immunological data from these studies, appropriate concentrations of these vitamins were chosen for the formal diet trials.

In these preliminary studies, it became apparent that mid-range concentrations of pyridoxine (60 mg/kg diet) afforded the greatest degree of immunological enhancement. Although variations in the immunological activity occurred in the folic acid trials, no consistent pattern emerged.

On the basis of these preliminary studies, concentrations of pyridoxine and folic acid were chosen for supplementation of the Abernathy and semi-purified diets.

At this point in time, we are in the midst of the formal diets studies, thus a rigorous statistical analysis of all the data is not possible. However, thus far, with respect to the pyridoxine trials, the immunological and disease resistance studies indicate that mid-range concentrations (60 mg/kg) of pyridoxine seem to enhance immunological activity and high concentrations > 100mg/kg may exert a negative effect on the immune response.

With respect to folic acid, there does not appear to be any difference in the immune response over the range of concentrations tested.

SUMMARY OF EXPENDITURES

	<u>84-45A</u>	<u>84-45B</u>
1. Salaries (including personnel and benefits)	\$16,869.58 ¹	\$21,880.16
2. Travel and transportation (including per diem)	688.41	2,447.88
3. Non-expendable equipment and material (greater than \$1,000 per item)	0.00	0.00
4. Expendable equipment and material (sensitive in nature)	0.00	0.00
5. Operations and maintenance (including computer services and publications)	4,168.53	19,611.19
6. Overhead	21,320.00 ²	14,939.35
7. The currently approved budget	85,387.80	161,747.00
8. Current budget period	8/1/85- 7/31/86	8/1/85- 7/31/86
9. Cumulative expenditures to date	75,304.94 ²	125,096.72 ³
¹ period 7/1/85 - 6/18/86		
² value is a close estimate, full billing and overhead costs not available at the time of report submission		
³ through 5/16/86		

REFERENCES

- Axelrod, A.E. and Traketellis, A.C. 1964. Relationship of pyridoxine to immunological phenomena. Pages 591-607 in R.W. Harris and I.G. Wool, editors. Vitamins and Hormones. Advances in Research and Applications.
- Banks, Joe L., Taylor, W.G. and Leek, S. 1979. Carrying capacity recommendations for Olympia Area National Fish Hatcheries. U.S. Fish and Wildlife Service, Abernathy Salmon Cultural Development Center, Processed Report, 57 pp.
- Banner, C.R., Rohovec, J.S. and Fryer, J.L. 1983. Renibacterium salmoninarum as a cause of mortality among chinook salmon in salt water. J. World. Maricul. Soc. 14:236.
- Beisel, W.R. 1982. Single nutrients and immunity. The American Journal of Clinical Nutrition 35(2): Feb. Supplement:417-468.
- Bell, G.R., Higgs, D.A., and Traxler, G.S. 1984. The effect of dietary ascorbate, zinc, and manganese on the development of experimentally induced bacterial kidney disease in sockeye salmon (Oncorhynchus nerka). Aquaculture. 36(4):293-311.
- Blalock, T.L., Thaxton, J.P. and Garlich, J.D. 1984. Humoral immunity in chicks experiencing marginal vitamin B-6 deficiency. J. Nutr. 114(2):312-322.
- Blazer, V.S. and Wolke, R.E. 1984. The effects of -tocopherol on the immune response and non-specific resistance factors of rainbow trout (Salmo gairdneri Richardson). Aquaculture 37(4):1-9.
- Brett, J.R., Shelbourn, J.E. and Shoop, C.T. 1969. Growth rate and body composition of fingerling sockeye salmon, Oncorhynchus nerka, in relation to temperature and ration size. J. Fish.

- Res. Board Can. 26(9):2363-2394.
- Buterbaugh, G.L. and Willoughby, H. 1967. A feeding guide for brook, brown and rainbow trout. The Progressive Fish-Culturist 29:210-215.
- Chatterjee, G.C. 1978. Nutrient deficiencies in animals: Vitamin C. Pages 149-176 in Miloslav Rechcigal, Jr., editor. Section E: Nutritional disorders, Volume II, Effects of Nutrient Deficiencies in Animals. CRC Handbook Series in Nutrition and Food. CRC Press, Inc., West Palm Beach, Florida.
- Colnago, G.L., Jensen, L.S. and Long, P.L. 1984. Effect of selenium and vitamin E on the development of immunity to coccidiosis in chickens. Poultry Science 63(6):1136-1143.
- Cunningham, A. and Szenberg, A. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14:599.
- Cunningham-Rundles, S. 1982. Effects of nutritional states on immunological function. J. Clinical Nutrition, 35(5):1202-1210.
- Debes, S.A. and Kirskey, A. 1979. Influence of dietary pyridoxine on selected immune capacities of rat dams and pups. The Journal of Nutrition 109:744-759.
- Durve, V.S. and Lovell, R.T. 1982. Vitamin C and disease resistance in channel catfish (Ictalurus punctatus). Can. J. Fish. Aquat. Sci. 39(7):948-951.
- Ellis, R.P. and Vorhies, M.W. 1976. Effects of supplemental dietary vitamin E on the serological response of swine to an Escherichia coli bacterin. JAVMA 168:231-232.
- Etlinger, H.M., Hodgins, H.O., and Chiller, J.M. 1976. Evolution of the lymphoid system. I. Evidence for lymphocyte heterogeneity

- in rainbow trout revealed by the organ distribution of mitogenic responses. J. Immunol. 116:1547.
- Fowler, L.G. and Burrows, R.E. 1971. The Abernathy salmon diet. The Progressive Fish-Culturist. 33:67-75.
- Fryer, J.L., and Sanders, J.E. 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. Microbial. 35:273.
- Halver, J.E. 1972. The vitamins. Pages 29-103 in Fish Nutrition. John E. Halver, editor. Academic Press, Inc., New York, New York.
- Hardy, R.W. 1979. Effect of dietary protein level on the pyridoxine requirement and disease resistance of chinook salmon. Pages 253-260 in Proceedings of the World Symposium on Finfish Nutrition and Fish Feed Technology, Vol. I, Hamburg, Germany, June 20-23, 1978.
- Heinseling, R.H., Nockels, C.F., Quarles, C.L. and Tengerdy, R.P. 1974. Protection of chicks against E. coli infection by dietary supplementation with vitamin E. Proc. Soc. Exp. Biol. Med. 146:279-283.
- Horwitz, W., editor. 1980. Official methods of analysis of the Association of Official Analytical Chemists. 13th edition. Assoc. Off. Anal. Chem., Washington, D.C., 1018 pp.
- Hublou, W.F. and Jones, I.W. 1970. Fish Commission of Oregon hatchery production summaries, 1960-67 broods of fall chinook, spring chinook, coho and chum salmon and steelhead trout. Fish Commission of Oregon, Division of Fish Culture, Processed Report, 39 p.
- Kaattari, S.L, and Irwin, M.J. 1985. Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell

- repertoires. Dev. Comp. Immunol. In press.
- Lee, D.J. and Sinnhuber, R.O. 1972. Lipid requirements. Pages 145-180 in Fish Nutrition J.E. Halver, editor. Fish Nutrition. Academic Press, Inc., New York, New York.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.S. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-276.
- Mahnken, C.V.W., Spinelli, J. and Waknitz, F.W. 1980. Evaluation of an alkane yeast (Candida sp.) as a substitute for fish meal in Oregon moist pellet inrainbow trout (Salmo gairdneri). Aquaculture 20:41-56.
- McCoy, C.R. 1976. Pages 621-630 in In Vitro Methods in Cell and Tumor Immunology. Bloom and David, editors. Academic Press, NY.
- McKinney, E.C., Ortiz, G., Lee, J.C., Sigel, M.M., Lopez, D.M. Epstein, R.S. and McLeod, T.F. 1976. Lymphocytes of fish: Multipotential or specialized? Pages 73-82 in Phylogeny of Thymus and Bone Marrow - Bursa Cells. Elsevier/NorthHolland Biomedical Press. Amsterdam, The Netherlands.
- Miller, N.W. and Clem, L.W. 1984. Microsystem for in vitro primary and seconday immunization of channel catfish (Ictalurus punctatus) leukocytes with hapten-carrier conjugates. J. Immunol. Methods. 72:367-379.
- Miller, N.W., R.C. Sizemore, and L.W. Clem. 1985. Phylogeny of lymphocyte heterogeneity: the cellular requirements for in vitro antibody responses of channel catfish leukocytes. J. Immunol. 134:2884-2888.
- National Academy of Sciences. 1973. Number 11, Nutrient

- requirements of trout, salmon and catfish. Nutrient Requirements of Domestic Animals. Academy of Science, Washington, D.C.
- Nelson, R.A. 1978. Effects of specific nutrient deficiencies in animals: Pantothenic acid. Pages 59-63 in Miloslav Rechcigal, Jr., editor. Section E: Nutritional Disorder, volume II, Effects of Nutrient Deficiencies in Animals. CRC Handbook Series in Nutrition and Food. CRC Press, Inc., West Palm Beach Florida.
- Nockels, C.R. 1980. The biological immune response - The effect of dietary vitamin E. Feedstuffs, June 16, pages, 22-23.
- Panda, B. and Combs, G.F. 1963. Impaired antibody production in chicks fed diets low in vitamin A, pantothenic acid or riboflavin. Proc. Soc. Exp. Biol. Med. 113:530-534.
- Rittenberg, M.B., and Pratt, K.L. 1969. Anti-triphenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 132:575.
- Sanders, J.E. 1979. Cell wall chemistry, deoxyribonucleic acid base composition and pathogenesis of the kidney disease bacterium in salmonid fishes. Ph.D. Thesis, Oregon State University, Corvallis, Oregon, 77p.
- Scott, M.L., Nesheim, M.C. and Young, R.J. 1976. Nutrition of the Chicken. M.L. Scott and Associates, Publishers, Ithaca, New York, 55 p.
- Siddons, R.C. 1978. Nutrient deficiencies in animals - folic acid. Pages 123-132. in Miloslav Rechcigal, Jr., editor. Section E: Nutritional Disorders, Volume II, Effects of Nutrient Deficiencies in Animals. CRC Handbook Series in Nutrition and

- Food. CRC Press Inc., West Palm Beach, Florida.
- Tittle, T.U., and Rittenberg, M.B. 1978. Expression of IgG memory in vitro to thymus dependent and thymus independent antigens. Cell. Immunol. 35:180.
- Wedemeyer, G.A., Meyer, F.P. and Smith, L. 1976. Environmental Stress and Fish Diseases. in S.F. Snieszko and H.R. Axelrod, editors. Diseases of Fishes, Book 5. T.F.H. Publications, Inc., Neptune, New Jersey.
- Wedemeyer, G.A. and Ross, A.J. 1973. Nutritional factors in the biochemical pathology of corynebacterial kidney disease in the coho salmon (Oncorhynchus kisutch). J. Fish. Res. Board Can. 30: 296-298.
- Wilgus, H.S. 1977. Disease, nutrition-interaction. Poultry Science 59:772-781.
- Wood, J.W. 1974. Disease of Pacific salmon, their prevention and treatment. State of Washington Department of Fisheries, Hatchery Division, Olympia, Washington, 82 p.
- Young, C.L., and Chapman, G.B. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salmo fontinalis). J. Fish Res. Board Can. 35:1234.